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*J Immunol* (1991) 146 (3): 893–898.
https://doi.org/10.4049/jimmunol.146.3.893

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0022-1767/91/1463-0893$02.00/0
THE JOURNAL OF IMMUNOLOGY
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Vol. 146, 893-898, No. 3, February 1, 1991
*Printed in U.S.A.*

# MAPPING THE EPITOPES OF NEUTRALIZING ANTI-HUMAN IL-3 MONOCLONAL ANTIBODIES

## Implications for Structure-Activity Relationship

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**The epitopes of neutralizing mAb were mapped in order to identify a receptor binding site on human IL-3 (huIL-3). To initiate this structure and activity analysis, four neutralizing mAb were selected on the basis of preventing rhuIL-3 stimulated proliferation of peripheral blood cells from a patient with chronic myelogenous leukemia (CML). In order to identify continuous epitopes, the neutralizing mAb were assayed in a solid-phase ELISA for their reactivity with either denatured rhuIL-3 or with the peptides generated by digestion of rhuIL-3 by using two different proteinases. Two of the neutralizing mAb recognized single fragments from both digestions. Amino acid (aa) sequence determination showed that these peptides overlap, defining a region of 22 aa (aa 29 to 50 of the mature rhuIL-3 protein). In a competition ELISA, the two continuous epitopes were shown to be linked to one another and to the two discontinuous epitopes, suggesting that all four neutralizing mAb bind to a discrete region of the IL-3 molecule, which might be involved in binding to the IL-3R.**

Cytokines play a vital role in the regulation of hemopoiesis. The T cell-derived lymphokine IL-3 is one of the growth factors that stimulates the proliferation and differentiation of hemopoietic precursor cells (1, 2). The cDNA for huIL-3[2] has been cloned and rhuIL-3 has been identified as a glycoprotein with a molecular mass ranging from 14.6 to 28 kDa (3, 4). The three-dimensional structure of huIL-3 is unknown. The IL-3R has been well characterized on murine cells (5–8), but little is known about the huIL-3R. Binding analysis with either human monocytes (9), basophils (10), the cell line KG-1 (11), or TF-1 (12) demonstrates that huIL-3 interacts with cells via a specific cell-surface receptor. Cross-linking experiments indicate that IL-3 binds to proteins with molecular mass of approximately 135 and 70 kDa (12, 13). Acute myeloblastic leukemia blasts and monocytes also have an intermediate size protein of 105 kDa (13). Some functional heterogeneity has been observed in huIL-3R. It is believed that a subclass of this receptor also binds human granulocyte-macrophage CSF (huGM-CSF) (13, 14). The signal transduction pathway of the huIL-3R is poorly understood.

A structural analysis of the IL-3 molecule could contribute to the understanding of the mode of action of IL-3 and the region interacting with the IL-3R is of special interest.

Here we describe a strategy to identify a receptor-binding site on the rhuIL-3 molecule. The approach was first to raise antibodies against the native IL-3 molecule and to select those which are able to neutralize IL-3 activity. Epitopes recognized by neutralizing mAb were subsequently localized by digestion of the rhuIL-3 molecule with different proteinases and by identification of the peptides. In this way, we could show that two out of four neutralizing mAb recognize continuous epitopes which are within a peptide fragment comprising aa residue 29 to 50 of the mature rhuIL-3 sequence. This sequence might therefore be an important site for the interaction of huIL-3 with its receptor.

### MATERIALS AND METHODS

*CML cells and IL-3 bioassay.* The CML bioassay has been described previously (15). Peripheral blood cells were obtained by leukophoresis from a CML patient, separated over Ficoll (Pharmacia), and cryopreserved in the vapor phase of liquid nitrogen in 10% DMSO and 20% heat-inactivated FCS (Boehringer, Mannheim, FRG). The cells were thawed and diluted in medium containing 0.01% (w/v) deoxyribonuclease I (Worthington Biochemicals, Frechold, NJ) to minimize cell agglutination. Washed cells were incubated at $10^6$ cells/ml in RPMI 1640 (GIBCO, Paisley, Scotland) containing 5% FCS at 37°C for 18 h before assay.

IL-3-specific mAb were tested for their ability to prevent IL-3 stimulated proliferation of CML cells. Cells were seeded at $2 \times 10^4$ per well in a 96-well microtiter plate (Falcon 3072, Becton Dickinson, NJ) in the presence of 15 U/ml of CHO-expressed rhuIL-3 and serial dilutions of hybridoma supernatants (1:4 to 1:256) or purified mAb (500 ng/ml to 0.01 ng/ml). After 68-h incubation at 37°C, 0.5 μCi [$^3$H]TdR (15 Ci/mmol, Amersham, Buckinghamshire, England) was added to each well and incubated for additional 4 h. Cells were harvested on filter papers (IH201 G-7, Inotech AG, Wohlen, Switzerland), which were washed, dried, and counted in a Beckman counter (LS 3801, Beckman Instruments, Palo Alto, CA) after addition of scintillation liquid (P/N 158735, Beckman Instruments).

CML neutralizing activity was defined as the concentration of mAb required to achieve 50% inhibition of CML cell proliferation.

*Lymphokines.* Purified, CHO-expressed rhuIL-3 for immunization was kindly provided by Genetics Institute, Cambridge, MA. Purified *Escherichia coli*-expressed rhuIL-2, CHO-expressed rhuGM-CSF, CHO- and *E. coli*-expressed rhuIL-3, as well as deglycosylated CHO-expressed rhuIL-3 were produced at Sandoz Pharma Ltd., Basle, Switzerland.

Received for publication June 26, 1990.
Accepted for publication November 6, 1990.
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[2] Abbreviations used in this paper: huIL-3, human IL-3; CML, chronic myelogenous leukemia; CHO, Chinese hamster ovary; huGM-CSF, human granulocyte-macrophage CSF; huIL-2, human IL-2; aa, amino acid.



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*Antibodies.* All antibodies used for isotype definition and detection in ELISA are described elsewhere.[3] Sheep polyclonal antibodies against CHO-expressed rhuIL-3 were produced in Sandoz Pharma Ltd., Basle, Switzerland.

*Generation of anti-rhuIL-3 mAb.* Murine mAb were prepared by immunizing BALB/c mice (Madörin, Füllingsdorf, Switzerland) with 50 μg purified, CHO-expressed rhuIL-3 given in a 1:1 mixture of CFA (Difco Laboratories, Detroit, MI) and PBS in the hind footpads. Five additional injections of 50 μg IL-3 in PBS were made in the footpad at 4-day intervals. One day after the final injection (day 21), the mice were bled from the retro-orbital venous plexus. The titers of the immune sera were determined in a solid-phase ELISA and one mouse with a high IgG titer was selected. On day 22, the lymph node cells of this mouse were fused (fusion 13, F13) with murine PAI-0 myeloma cells (16), essentially as previously described (17). One month later, another mouse was boosted with 25 μg rhuIL-3 in PBS into the footpad daily for 4 days. One day after the final injection, lymph node cells or spleen cells were fused with the PAI-0 cells (F14, F15 respectively). Supernatants from wells positive for hybridoma growth were screened in a solid-phase ELISA. The cells producing anti-rhuIL-3 antibodies were cloned by limiting dilution (18) and adapted to serum-free medium. Selected mAb were purified (19) and biotinylated as previously described.[3]

*Enzyme immunoassays: ELISA.* All general ELISA steps (coating, blocking, washing, and detection procedures) were performed as described.[3] Briefly, after coating of the different samples to the solid-phase of 96-well microtiter plates, the plates were blocked with PBS containing 2% BSA and incubated with the different mAb samples. Bound mAb were detected with goat anti-mouse IgG-biotin followed by streptavidin-alkaline phosphatase. ELISA were also used for isotype determination and quantification of the mAb.[3]

*Specificity ELISA.* Purified neutralizing and nonneutralizing mAb (500 ng/ml) were assayed for binding to solid-phase coupled IL-2, GM-CSF, *E. coli*- and CHO-expressed rhuIL-3 (native and denatured) as well as for binding to IL-3 digests generated by treatment of rhuIL-3 with proteinase Lys-C or Glu-C (see below) at the concentrations indicated (39 to 2,500 ng/ml).

The mAb were also tested, in a similar ELISA, for binding to the fractions containing the reverse phase HPLC separated IL-3 peptides. Briefly, the peptide content of each HPLC-fraction was adjusted to a concentration of 10 μM so that each dilution represents an equal amount of peptide. Serial dilutions of these peptides (1:10 to 1:2560) were coated to the solid-phase of an microtiterplate and subsequently incubated with the mAb (500 ng/ml). For each fraction tested, the dilution required for 50% maximal binding of each mAb was determined. To control the binding of the different peptides to the ELISA plates, each HPLC fraction was also incubated with a polyclonal anti-rhuIL-3 antibody preparation.

*Relative affinity of mAb.* The relative affinities of IL-3 specific mAb were determined in ELISA. Sheep anti-rhuIL-3 IgG (5 μg/ml) was coated overnight at 4°C on ELISA plates, which were then blocked with 2% BSA in PBS, washed, and incubated with CHO-expressed rhuIL-3 (500 ng/ml). Each mAb to be tested was added (serial dilution of 50,000 ng/ml to 0.0256 ng/ml), incubated overnight at 4°C, and bound mAb was detected with streptavidin-alkaline phosphatase. The relative affinity of each mAb is defined as the concentration required to produce half-maximal binding. Normalized neutralizing activity was calculated as the ratio of relative affinity to the CML neutralizing activity.

*Competition ELISA.* Competition experiments were performed to define the spatial relationship between epitopes of different mAb. Plates were coated with 100 ng/ml CHO-expressed rhuIL-3. In an initial experiment, different concentrations (0.5 to 10,000 ng/ml) of each biotinylated mAb were added to the coated plates and detection was performed with streptavidin-alkaline phosphatase in order to determine the amount of each biotinylated mAb required to produce an absorbance of one. These concentrations were used in all subsequent experiments. A competition ELISA was then performed to determine whether two mAb could compete for binding to the solid phase coupled rhuIL-3. Fifty microliters of serial dilutions of competitor (unbiotinylated mAb) were preincubated with the IL-3-coated plates, and, after 1 h, 50 μl of the predetermined amount of biotinylated mAb were added. Bound biotinylated mAb in the presence of competitor was detected after a further 1-h incubation. The ratio of competitor to biotinylated mAb required for 50% inhibition of binding of biotinylated mAb to solid-phase coupled IL-3 was calculated.

[3] **Zenke, G., U. Strittmatter, R. Tees, E. Andersen, B. Fagg, H. P. Kocher, and M. H. Schreier.** A cocktail of three monoclonal antibodies significantly increases the sensitivity of an enzyme immunoassay for human granulocyte-macrophage colony-stimulating factor. *J. Immunoassay. In press.*

*Reduction, alkylation, and proteinase treatment of rhuIL-3.* For reduction, 2.5 mg lyophilized CHO-expressed rhuIL-3 were dissolved in 1 ml guanidine-Tris buffer (6 M guanidine-hydrochloride, 0.45 M Tris-hydrochloride, pH 8.0). Five micrograms of dithiothreitol were added and the reaction mixture was kept under an atmosphere of argon at room temperature in the dark for 2 h. Alkylation was performed by addition of 100 μl 4-vinylpyridine dissolved in 400 μl isopropanol to the solution containing the reduced protein. Incubation under argon at room temperature in the dark was continued for an additional 2 h followed by dialysis of an aliquot against 50 mM Tris-hydrochloride, pH 8.0 buffer for subsequent cleavage with endoproteinase Lys-C (Boehringer) and 50 mM ammonium acetate, pH 4.0 buffer for cleavage with endoproteinase Glu-C (Boehringer), respectively. Proteolytic cleavage was carried out by addition of 5% enzyme (w/w) to the reduced and alkylated protein in the respective buffers and incubation at 37°C for 4 h.

The peptides generated by proteolytic cleavage were separated on an Applied Biosystems 130A gradient HPLC system equipped with a Brownlee RP-300 C8 (2.1 × 30 mm) column. Buffer A consisted of 0.1% trifluoroacetic acid in water, buffer B of 0.08% trifluoroacetic acid, 70% acetonitile, and water. A linear gradient from 0% to 60% buffer B in 20 min followed by 60% to 100% buffer B in 5 min was used. Eluting peptides were manually collected in Eppendorf tubes.

*The aa sequence determination.* Each fraction was reapplied on identical HPLC columns for appropriate separation of the peptide before aa sequencing.

Peptides, which were isolated after the second-run HPLC, were applied to Polybrene (Sigma)-treated glass fiber disks and subjected to aa sequence determination on an Applied Biosystems 470A protein sequencer equipped with an on-line PTH derivative detection system according to standard procedures.

## RESULTS

*Selection of neutralizing mAb.* A total of 42 hybridomas producing rhuIL-3-specific antibodies were identified in ELISA (data not shown), cloned, and the supernatants tested in the CML bioassay for the capacity to neutralize IL-3 activity. All mAb exhibiting neutralizing activity were purified from serum-free culture supernatants and characterized. The inhibition of CML cell proliferation by four purified mAb is shown in Table I. F14-570 and F14-746 were the most effective neutralizing mAb, indicated by a low amount of mAb required for inhibition of proliferation to half maximum. The two other mAb F13-267 and F15-216 neutralized IL-3 activity at about 70- to 245-fold higher concentrations. The relative affinity of each neutralizing mAb was defined in ELISA (Table I). This was performed by using IL-3 bound to solid-phase coupled polyclonal anti-IL-3 antibodies in order to minimize conformational changes of IL-3, which can occur through direct coupling of the Ag to plastic (20). The three mAb F14-570, F14-746, and F15-216 have approximately three- to fourfold higher relative affinity compared with F13-267. A nonneutralizing mAb F13-947 had no effect on IL-3 activity when tested at 166

TABLE I
*Neutralizing activities and relative affinities of anti-rhuIL-3 mAb*

| mAb | Neutralizing Activity[a] (ng/ml) | Relative Affinity[b] (ng/ml) | Normalized Neutralizing Activity[c] |
|---|---|---|---|
| F14-570 | 200 ± 100 | 5 | 40 |
| F14-746 | 200 ± 50 | 7 | 29 |
| F13-267 | 14,000 ± 6,000 | 20 | 700 |
| F15-216 | 49,000 ± 22,000 | 5 | 9,800 |
| F13-947 | >166,000 | 24 | — |

[a] The CML assay was performed as described in *Materials and Methods.* mAb were titrated in the presence of 15 U/ml (5 ng/ml aglycoprotein) CHO-expressed rhuIL-3 in three independent experiments. Concentrations (mean ± SD) of mAb required for 50% inhibition of proliferation are shown.

[b] Relative affinity of mAb determined in ELISA.

[c] Normalized neutralizing activity = neutralizing activity/relative affinity.

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μg/ml even though it has a similar relative affinity compared with F13-267. Normalized neutralizing activity (CML neutralizing activity/relative affinity) of these mAb demonstrate that the strongest neutralizing mAb are F14-570, F14-746, and F13-267, whereas F15-216 inhibits IL-3 proliferation less efficiently (Table I).

The specificity of the neutralizing mAb was determined by using ELISA plates coated with CHO-, *E. coli*- and deglycosylated CHO-expressed rhuIL-3 in order to distinguish between mAb recognizing the carbohydrate or the protein moiety of the IL-3 molecule (data not shown). The mAb bound similarly to each rhuIL-3 preparation assayed, indicating that they all were specific for protein determinants. They showed no binding to the unrelated lymphokines rhuIL-2 and rhuGM-CSF. All mAb belonged to the IgG1 subclass and have a κ-L chain, except F13-267 which has IgG2b H chain and a λ-L chain.

*Distinction between continuous and discontinuous epitopes of neutralizing mAb.* Our strategy for structure and activity relationship analysis is based on the assumption that identification of the region on the surface of the IL-3 molecule recognized by neutralizing mAb could indicate a region involved in binding to the IL-3R. Here we concentrate on the identification of continuous epitopes, which can be easily mapped. Having identified neutralizing mAb we first needed to determine whether they recognized continuous or discontinuous epitopes of IL-3. The neutralizing mAb were therefore tested for direct binding to native and denatured CHO-expressed rhuIL-3 in ELISA (Fig. 1). Three mAb (F13-267, F14-570, and F14-746) showed a decreased binding to denatured IL-3 compared with native IL-3 (respectively Fig. 1, *A*, *B*, and *C*). No clear discrimination between continuous and discontinuous epitopes was thus possible for these mAb. One mAb (F15-216) exhibited similar binding curves (Fig. 1*D*), suggesting that this mAb recognizes a continuous epitope.

In order to localize the epitopes of the neutralizing mAb, binding of the mAb to IL-3 peptides was analyzed in the same ELISA. Peptides were generated by digestion of rhuIL-3 with two different proteinases, Lys-C cutting C-terminal to lysine and Glu-C cutting C-terminal to glutamic acid. These enzymes were selected because of the position of potential cleavage sites present within the deduced aa sequence of the mature rhuIL-3 molecule, which should yield peptides of suitable size (3). First, binding was analyzed with the mixture of peptides bound to the solid phase of an ELISA plate (Fig. 1). The binding results with the neutralizing mAb showed that two mAb (F13-267 and F15-216) clearly recognized peptides in both digestion mixtures, indicating that they have continuous epitopes (Fig. 1, *A* and *D*). F13-267 showed a weaker reactivity with the mixtures than F15-216, probably due to the lower relative affinity for IL-3 (Table I). The two other mAb F14-570 and F14-746 failed to bind to either of the peptide digests suggesting that their epitopes were completely disrupted by digestion of rhuIL-3 with either Lys-C or Glu-C (Fig. 1, *B* and *C*).

*Localization of continuous epitopes of neutralizing mAb.* The individual peptides generated by Lys-C and Glu-C digestion of rhuIL-3 were separated by reverse phase HPLC and each fraction was tested for reactivity with the neutralizing mAb in an ELISA (Fig. 2). In agreement with the findings in the previous experiments with the digestion mixtures, the two mAb F14-570 and F14-746 did not react with any of the peptides (data not shown). Binding analysis with the individual peptides from the Lys-C digestion showed that only HPLC fraction number 15 was recognized by mAb F13-267 (Fig. 2*B*) and by mAb F15-216 (Fig. 2*C*). In the case of the Glu-C digestion, two HPLC fractions (numbers 10 and 11) were recognized by the mAb F13-267 (Fig. 2*E*) and F15-216 (Fig. 2*F*). Reactivity with two fractions could indicate that the Glu-C digestion was incomplete and/or that the peptides were not completely separated. A polyclonal anti-rhuIL-3 antibody bound to each HPLC fraction indicating that all peptides were effectively coupled to the solid phase of the ELISA plates (data not shown).

The fractions binding to the neutralizing mAb were reapplied to the same HPLC column for separation before

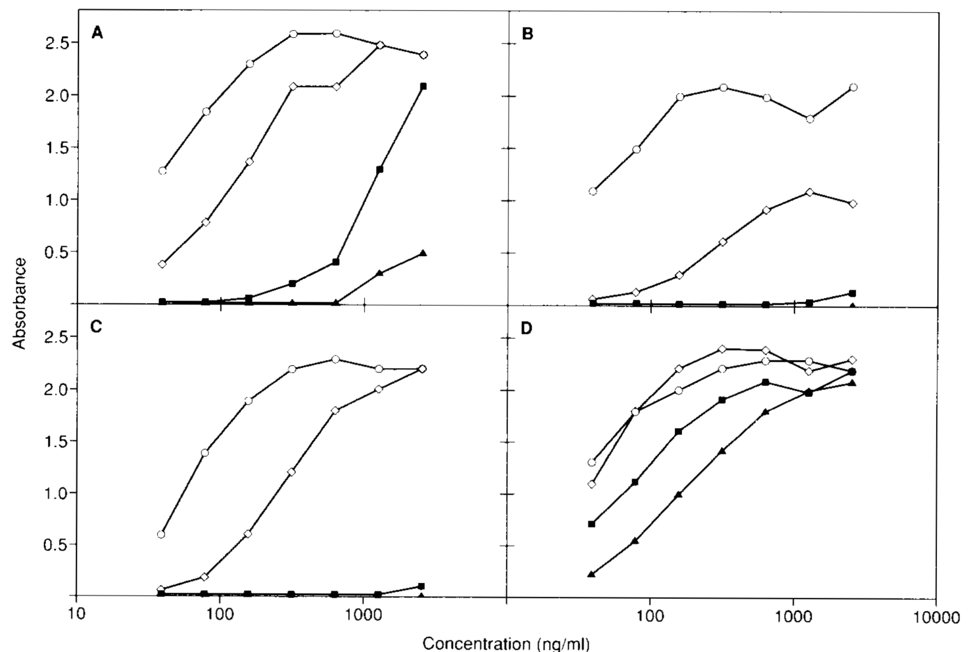


*Figure 1.* Distinction between continuous and discontinuous epitopes of neutralizing mAb in ELISA. Binding of neutralizing mAb to solid-phase coupled native (○), denatured (◇), and Lys-C (■) or Glu-C (▲) digested CHO-expressed rhuIL-3 in solid-phase ELISA. The plates were coated with the indicated concentrations of rhuIL-3 (native or denatured) or digestion mixture of IL-3 peptides and incubated with 500 ng/ml of the following mAb (*A*, F13-267; *B*, F14-570; *C*, F14-746; and *D*, F15-216).

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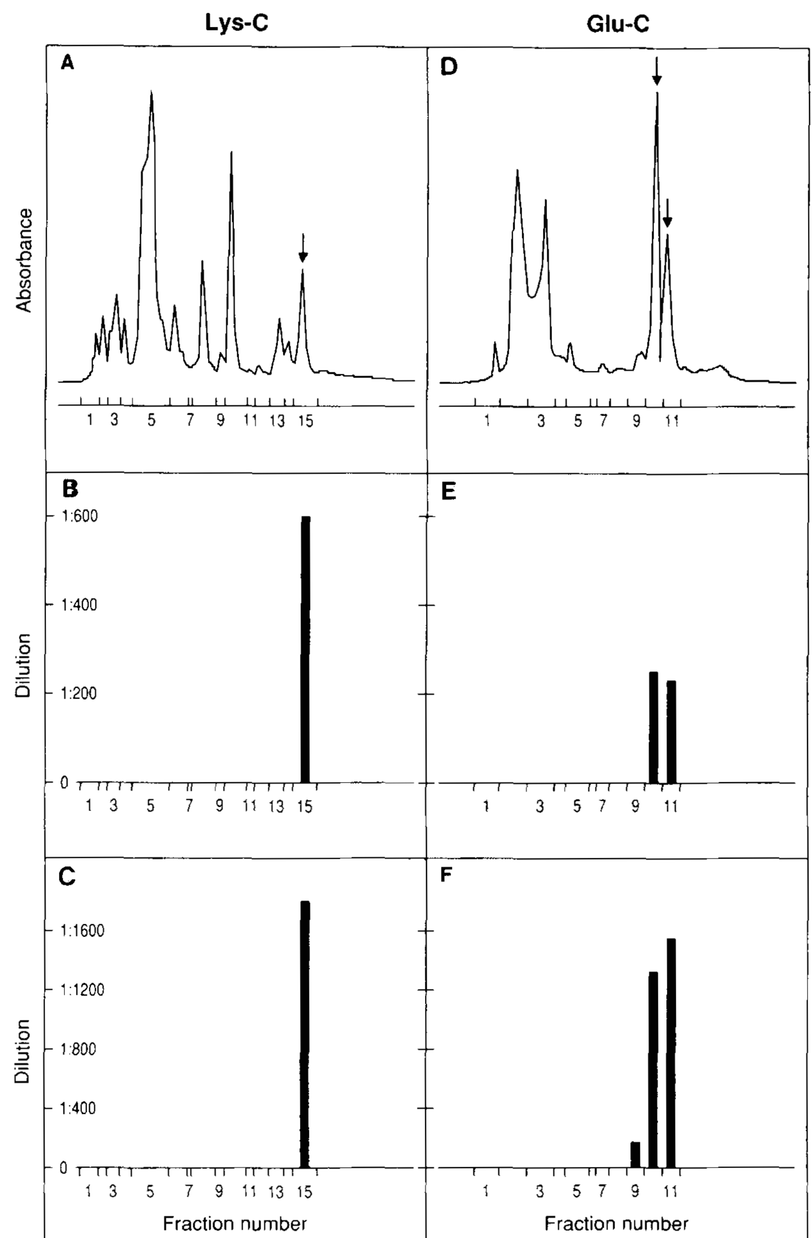


*Figure 2.* Identification of IL-3 peptides containing linear epitopes of two neutralizing mAb in ELISA. Reverse phase HPLC chromatogramm of Lys-C (*A*) and Glu-C (*D*) digested rhuIL-3. The eluted peptides were collected in fractions and all concentrations were set to 10 μM as described in *Materials and Methods*. *Arrows* indicate the HPLC fractions analyzed for aa sequencing. The reactivity of mAb F13-267 with the solid-phase coupled peptides contained in the HPLC fractions of Lys-C digest (*B*) and Glu-C digest (*E*), and of mAb F15-216 with Lys-C digest (*C*) and Glu-C digest (*F*) is shown. The dilution of HPLC fractions required for half-maximal binding of mAb are shown. No *bars* are shown if the dilution required is less than 1:10. The mAb F14-570 and F14-746 did not show any binding to the HPLC fractions.

aa sequence determination. The peptide contained in fraction 15 of the Lys-C digest, extended from aa 29 to 66 (Fig. 3). A common peptide of 28 aa, ranging from aa 23 to 50, was identified in both fractions 10 and 11 of the Glu-C digestion. Sequencing data revealed that Glu-C had failed to cleave the molecule after the glutamic acid residue at position 43. The two identified peptides overlap defining a region of 22 aa, from aa 29 to 50 of the mature IL-3 protein, which contains the epitopes of two neutralizing mAb (Fig. 3).

*Mutual competition analysis of the neutralizing mAb.* As the four neutralizing mAb do not show more than a fourfold difference in relative affinity (Table I), we attempted to define the linkage between the epitopes of these mAb in a competition ELISA. For each pair of mAb tested, the ratio of unlabeled mAb to labeled mAb required for 50% inhibition of binding of labeled mAb to the solid-phase coupled rhuIL-3 was calculated (Table II). No inhibition was observed with a nonneutralizing mAb (F13-947). The values on the diagonal indicate that each mAb could compete with itself. The other values to the right of this diagonal show that, in all cases, competition for binding to the solid-phase coupled IL-3 occurred, because one mAb could inhibit the binding of the second mAb. The results were less clear when mAb F14-570 and F14-746 were used as unlabeled competitors. No inhibition was observed when they were tested in combination with the labeled mAb F13-267 or F15-216. However, when labeled mAb F14-570 and F14-746 were used in the presence of the unlabeled competitors F13-267 or F15-216, mutual competition was observed. The results taken all together indicate that the epitopes of all neutralizing mAb are probably closely related suggesting that a discrete region of the IL-3 molecule might be involved in receptor binding.

## DISCUSSION

The use of antibodies in structure-activity relationship studies of proteins of unknown structure has been extensively described. For a large number of cytokines, anti-

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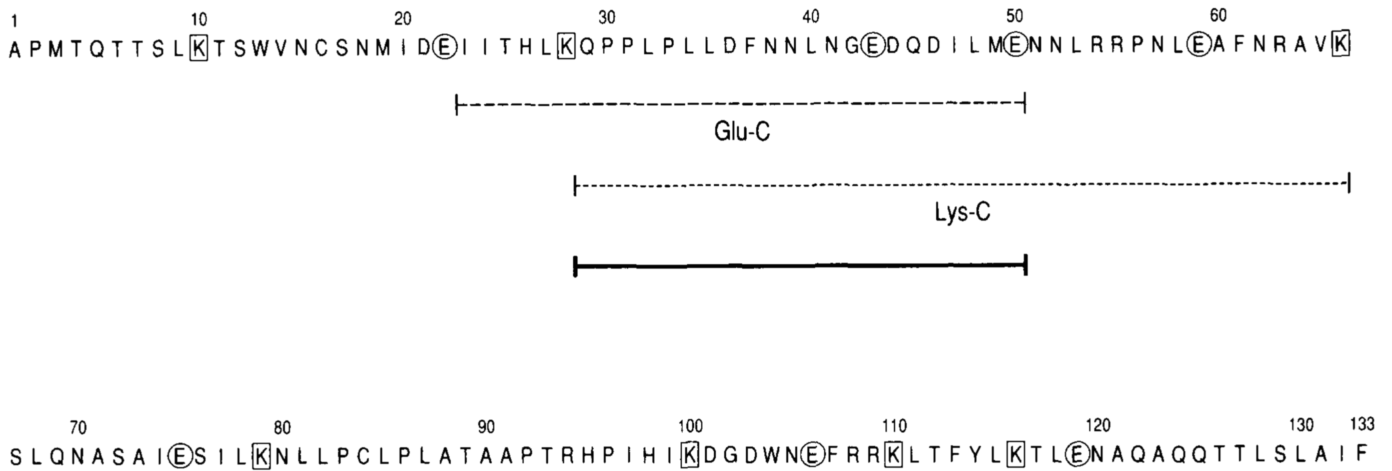


*Figure 3.* The aa sequence of mature huIL-3 (3). The potential cleavage sites for Lys-C, cutting C-terminal to K (*boxed*) and Glu-C, cutting C-terminal to E (*circled*) are represented. The IL-3 peptides recognized by the neutralizing mAb F13-267 and F15-216 are *underlined* (Glu-C and Lys-C) and encompass an overlapping region of 22 aa (*bold line*).

TABLE II
*Mutual competition of neutralizing mAb in ELISA*[a]

| Biotinylated mAb | Unlabeled Competitor | | | | |
|---|---|---|---|---|---|
| | F14-570 | F14-746 | F13-267 | F15-216 | F13-947[b] |
| F14-570 (200 ng/ml)[c] | 1[d] | 1 | 10 | 7 | >200[e] |
| F14-746 (100 ng/ml) | 2 | 2 | 50 | 29 | >200 |
| F13-267 (300 ng/ml) | >100 | >100 | 6 | 1 | >100 |
| F15-216 (50 ng/ml) | >400 | >400 | 330 | 4 | >400 |
| F13-947 (500 ng/ml) | >50 | >50 | >50 | >50 | 3 |

[a] Binding of biotinylated mAb to solid-phase coupled CHO-rhuIL-3 (100 ng/ml) in the presence of competitor (unlabeled mAb).
[b] F13-947 was used as a control anti-rhuIL-3 mAb with no neutralizing activity.
[c] Concentration of biotinylated mAb required to produce an absorbance of one in the absence of competitor as defined in *Materials and Methods*.
[d] Ratio of competitor to biotinylated mAb required for 50% inhibition of biotinylated mAb binding.
[e] In cases where a concentration could not be defined, the highest concentration used in the experiment is indicated with >.

bodies were raised against synthetic peptides (e.g., IL-1 (21, 22); IL-2 (23, 24); murine IL-3 (25, 26); erythropoietin (27), and IFN-$\gamma$ (28–31)). The use of antipeptide antibodies has the advantage that the regions of the protein, and thus the epitopes are defined. The peptides are chosen on the basis of structure predictions, which indicate that they are located on the surface, and therefore probably involved in the function of the protein.

We have used the native protein as immunogen and mapped the epitopes of mAb, which neutralized the activity of huIL-3. The mapping of discontinuous epitopes can be done only by fairly difficult techniques such as chemical modification of free and antibody-bound protein Ag (32) and/or limited proteolysis of Ag-antibody complex (33, 34). Therefore, we selected neutralizing mAb, which recognized continuous epitopes by testing them for reactivity with native vs. denatured rhuIL-3. Interestingly, nearly all anti-rhuIL-3 mAb (42 in total) still bound to the denatured IL-3 (our unpublished observations). This finding suggests that the denatured rhuIL-3 could be partially refolded, although the denatured molecule clearly differed from the native form in retention time on reverse-phase HPLC (data not shown). The binding data shown in Figure 1 indicated that mAb F15-216 recognizes a continuous epitope. However, they did not give sufficient information to determine the nature of the remaining epitopes. Therefore, in a further step, the denatured rhuIL-3 was digested with two different proteinases. The aa sequencing of immunoreactive peptides demonstrated that the continuous epitopes of both mAb, F13-267 and F15-216, were localized within a 22-aa overlapping region (aa 29 to 50) of huIL-3 (Fig. 3). The inability of the two mAb F14-570 and F14-746 to bind any of the digestion mixtures or separated peptides excludes a possible contamination of the immunoreactive peptide fractions with intact rhuIL-3. Furthermore, the retention time of these fractions clearly differed from that observed with undigested rhuIL-3 (data not shown). These mAb might recognize discontinuous epitopes. However, alternative explanations are that the two proteinases, Lys-C and Glu-C, cut directly within the epitopes or that the epitopes are within the fragments but these do not assume the same conformation as the native molecule. The observation that all four mAb could effectively compete with one another in a competition ELISA indicates that the epitopes of all neutralizing mAb might be related to one another. These results suggest that a discrete region of the IL-3 molecule interacts with the receptor. This conclusion is based on the assumption that the epitopes of the neutralizing mAb are directly included in the receptor-binding site, however, neutralization could also occur by steric hindrance (35, 36) or by induced conformational changes (37). This question could be clarified by site-directed mutagenesis within the continuous epitopes of the neutralizing mAb and subsequent testing of the variants for biologic activity and binding to the mAb.

It has been shown that mAb of different epitope composition are neutralizing, demonstrating that the active site can be larger or more complex than a single antigenic epitope (35). Thus linear epitopes might include only a part of the receptor-binding site. The information that the two neutralizing mAb F14-570 and F14-746 do not bind to any of the peptides suggests that additional sites of the IL-3 molecule are involved in receptor binding. This is supported to some extent by the recent cloning of a component of the murine IL-3R (8). This receptor chain binds to IL-3 with low affinity, suggesting that the IL-3R is a complex of more than one subunit and would therefore resemble the IL-2R. X-ray analysis together with structure-activity relationship studies of the IL-2 mole-

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cule also demonstrated that this factor interacts with the IL-2R through several contact sites (38, 39).

In the present study, we have localized the epitopes of two neutralizing mAb, directed against the native huIL-3 molecule, within a 22-aa peptide of the huIL-3 molecule. This information has provided the basis for our current site-directed mutagenesis experiments, aimed at the identification of the biologically relevant sites of huIL-3.

*Acknowledgments.* We thank Dr. M. Heitzman for the deglycosylated IL-3 and Dr. R. Papoian for the sheep polyclonal anti-rhuIL-3 antibodies.

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